A STUDY OF THE MECHANISM OF ACTIVATION AND NUCLEAR BREAKDOWN IN THE CHAETOPTERUS EGG [1]

LESTER GOLDSTEIN [2]

Department of Zoology, University of Pennsylvania, Philadelphia 4, Pa., and the Marine Biological Laboratory, Woods Hole, Massachusetts

The phenomenon of stimulation and response has been studied in a wide range of biological systems by a considerable number of investigators. Because of the many advantages of marine egg material, numerous workers have devoted their efforts to an examination of stimulation or activation[3] as it occurs in this material. These workers have believed that the phenomenon of stimulation and response is based on a fundamental cellular mechanism, the response to which, in eggs, is manifested by the initiation of some aspect of development. Some have limited their investigations to the maturation process, or more strictly the breakdown of the germinal vesicle nucleus, since they reasoned that this response was not complicated by as many factors as is cleavage.

The investigations reported here were concerned with nuclear breakdown in the immature eggs of the annelid *Chaetopterus pergamentaceus*. This material is especially favorable for study because of a number of characteristics not present in other available forms. For one thing, 100% of the eggs will show nuclear breakdown in a large majority of the worms, thereby insuring rather good reproducibility of results. Furthermore, nuclear breakdown occurs spontaneously in sea water, eliminating the necessity of artificial activation. These factors were in addition to the advantages existing also in other material—such as an easily detectable response and a short time period between stimulation and response.

Until 1950 only one paper, that of Allyn (1912), had appeared in which an attempt was made to explain, by experimental procedures, nuclear breakdown in Chaetopterus eggs. However, some work has been done on eggs of other forms. This earlier research on the process of nuclear breakdown includes the investigation of the effects of pH and ions on Pomatocerus eggs by Hörstadius (1923); a considerable amount of work, mostly concerned with effects of salts, by Dalcq (1928) and Pasteels (1935) on a variety of forms; experiments by Heilbrunn and Wilbur (1937) on the role of calcium ions in the initiation of nuclear breakdown in Nereis eggs; some work by Scheer and Scheer (1947) on the effects of

[3] In order to avoid any possible confusion with specific embryological terms, activation is defined here as any response to stimulation (either in egg cells or in other types of living cells). In this instance, it is the initiation of the cellular activity leading to nuclear breakdown.

drugs and ions in the activation of Urechis eggs; and some investigations by LeFevre (1945) concerning the effects of picric acid on the activation of Nereis eggs. There exists, of course, a much larger literature on fertilization and artificial parthenogenesis, phenomena which are related—in terms of activation—to the process of nuclear breakdown in oöcytes. The above mentioned papers, as well as reviews by Lillie (1941), Tyler (1941), Runnström (1949), Danielli (1951), and Heilbrunn (1952), may be consulted for a more extensive bibliography on the various aspects of activation in general.

So long as the eggs of Chaetopterus are retained within the parapodia of the worm their large (germinal vesicle) nuclei remain intact. This is apparent from the fact that if the eggs are examined within the first 5 minutes after they are shed, the large nucleus with its membrane can be seen in 100% of the eggs. Within 7 or 8 minutes after the eggs reach sea water, the nuclear membrane disintegrates and some of the nuclear material migrates to the periphery of the egg where the metaphase spindle of the first maturation division is formed. It has been the aim of this research (some of which is reported here) to answer, in physical and chemical terms, three questions relating to the phenomenon of nuclear breakdown as it occurs in the immature Chaetopterus egg.

Since the stimulus for the breakdown of the nucleus occurs as a result of the release of the egg from the animal into sea water, the first question posed was— is the initial activation a result of a direct external stimulus or is it a result of a release from an inhibition? As noted earlier in a preliminary note (Goldstein, 1950), the activation is apparently produced by the release of the egg from an inhibitory environment. This conclusion was based on the observations that nuclear breakdown would occur in the presence of any one of the four common cations (Na, K, Ca, and Mg), in the presence of any of several anions (Cl, NO_3, HCO_3, and Br), in sucrose solutions, in hypertonic and hypotonic sea water, at any pH between 3.5 and 9.0, and in the absence of oxygen. Thus, it was concluded that no factor in the sea water is responsible for the stimulus, but rather that activation results from a release from an inhibition. This view agrees with the opinion of Allyn (1912) and the results obtained by Scott and LeBaron (1950). The foregoing information led to the formulation of the second question, namely, what is the nature of the inhibitor present in the ovarian environment? Though a substantial amount of work has been done, most of the data concerning the inhibitor have been negative. At this time it is possible to say with assurance only that the inhibitor is heat labile and of a small enough molecular size to be dialyzable. (In a previous report (1950) it was stated that CO_2 might possibly be at least partially responsible for the inhibition. More recent and more extensive examination has indicated that this position is no longer tenable.) The third question is the one with which this paper is primarily concerned: What is the intracellular mechanism that produces the nuclear breakdown?

As a result of some early experiments, which revealed that calcium played an essential role in the activation process (cf. Goldstein, 1950), I was prompted to employ the colloid chemical theory of stimulation of Heilbrunn (1952) as a working basis for experimental design. This theory states, in part, that any stimulus applied to the cell will release calcium ions from a bound state, and that the calcium ions will then react with the protoplasmic colloids to produce the specific

cellular response. This view holds, moreover, that the mechanism is analogous to that involved in the clotting of blood. The approach employed here was that of "dissecting" the mechanism in such a manner that each individual step might be examined separately, following—to some extent—the approach used in interpreting the steps in blood clotting reactions.

I am grateful to Dr. L. V. Heilbrunn for suggesting this problem and for his willing encouragement, help and interest during the course of the work.

Material and Methods

Male and female Chaetopterus worms, obtained from the Woods Hole, Mass., area, were kept in separate fingerbowls under running sea water. Worms kept in this fashion did not often shed gametes in the laboratory and could frequently be maintained in good condition for as long as two or three weeks. Eggs and sperm were obtained by cutting as many posterior parapodia from the worm as would supply sufficient material for a particular experiment, and then the parapodia were cut open (into the appropriate solution) to release the gametes. In those instances where the eggs were shed directly into solutions other than normal sea water, the worms were first rinsed with distilled water and dried externally with filter paper in order to prevent contamination with substances in the sea water. In order to be able to carry out certain experimental procedures within two minutes after the eggs were shed, some preliminary screening was carried out. As a result, a few deviations from the commonly accepted handling of Chaetopterus eggs were employed. Thus, it was determined that straining and washing of the eggs were ordinarily unnecessary for normal nuclear breakdown, once the parapodia were cut open in sea water. (Usually between 10 and 20 parapodia were cut into a dish containing 15–20 ml. of solution.) The processes under study were apparently unaffected by any extraneous material from the worm. Furthermore, it has also been observed that virtually no degree of crowding of the eggs would affect the progress of nuclear breakdown.

Two criteria for determining normality of the eggs were used. First, the eggs shed into sea water were expected to yield nuclear breakdown in 100 of 100 eggs. This expectation was realized in 90–98% of the worms. The second criterion, generally observed, was that nuclear breakdown would occur within 7 or 8 minutes after the eggs were placed in sea water. Counts were made of at least 100 eggs from a dish, except in experiments where it was necessary to make rapid counts, in which case only 50 eggs were counted. In all the work reported here the data were of such a nature as to warrant no further statistical treatment. To examine and count the eggs it was necessary to remove a drop of egg suspension to a slide and flatten the eggs with a coverslip. This was done in order to determine the presence or absence of the nucleus (but even without flattening it was often possible to see the clear polar region of the first maturation metaphase spindle).

In many experiments, though not in all, the normalcy of the eggs before and after treatment was determined by fertilizing the eggs upon their return to normal sea water. Fertilization (and subsequent cleavage) was taken as an indication of viability in a sufficient proportion of the experiments in a series to reveal whether or not a particular experimental technique involved irreversible damage. Experi-

ments with various types of inhibitors were considered valid only if the inhibition were reversible.

In utilizing CO_2 the following procedure was employed. The gas was bubbled into the solution through fine bolting cloth covering the opening of a glass tube leading from the tank. Since the gas was bubbled through under relatively low pressures, it was not possible (with the equipment available) to determine accurately the quantities of gas being delivered, and only rough estimates were made. Indeed, more exact estimates were hardly necessary and, in view of the fact that the material behaved in consistent fashion, the conclusions drawn from the experiments are believed to be valid.

Measurements of pH were all made with a Beckman pH meter.

In using the hand centrifuge for determinations of the rigidity of the cell cortex, the technique described by Wilson (1951) was employed.

Sea water modifications were made up according to Table I and were adapted, with certain alterations, from the analyses provided by Wattenberg (1938).

TABLE I

Sea water modifications

	NaCl	KCl	CaCl₂	MgCl₂	MgSO₄	NaHCO₃
Artificial sea water	410	8.85	10.2	20.7	29.6	2.0
Ca-free sea water	428	9.3	——	20.7	29.6	2.0
Ca- and Mg-free sea water	454	10.1	——	5.7 (K₂SO₄)	24.1 (Na₂SO₄)	2.0
Mg-free sea water	426	9.7	10.2	5.7 (K₂SO₄)	24.1 (Na₂SO₄)	2.0

Figures represent ml. of molar stock solutions of the various salts. In each case, distilled water is added to make the final volume one liter.

The sodium citrate solution used throughout the investigation consisted of: 50 parts of 0.35 *M* sodium citrate plus 50 parts of Ca-free sea water. This solution will hereafter be called the *citrate solution.*

In analyzing for proteolytic activity, the method of Anson (1938) for trypsin determination with a hemoglobin substrate was followed as closely as possible. Incubation was carried out for one hour at 25° C. and the Folin-Ciocalteau reagent was added to color the reaction products.

RESULTS AND DISCUSSION

Cortical changes

Wilson (1951) has described physical changes in the protoplasmic cortex of Chaetopterus eggs during the mitotic cycle and these have been related to Heilbrunn's theory of stimulation and response. If the theory is correct, we should expect that somewhat similar changes would occur if the eggs were activated to produce nuclear breakdown. For reasons that will become evident shortly, a study was made of cortical changes in the maturing egg only during the first few minutes following activation (see Wilson for method).

Eggs were shed into artificial Ca-free sea water, which was somewhat inhibitory to nuclear breakdown.[4] *Two* minutes after the eggs were placed in the Ca-free sea water they were centrifuged at various rates for one minute, removed from the centrifuge tube, placed on a slide with a coverslip and examined for the presence of cortical granules.   The absence of granules in the cortical region of the cell is taken as an indication that the cortex had become less rigid and could no longer resist the centrifugal forces applied.

Table II shows that the percentage of eggs in which the cortical granules were moved by centrifugation was always in agreement with the percentage of eggs showing nuclear breakdown as observed at 8 minutes after the eggs were shed. (The observations on the cortex were made at about 4 minutes after shedding— before the nuclei have broken down.)   Further evidence was offered by the fact that if the eggs were centrifuged following nuclear breakdown, the eggs which retained intact nuclei always had a more rigid cortex.   This alone, however, could not be considered seriously as proof of a correlation between cortical change and nuclear breakdown since it was impossible to control for the presence or absence of the nucleus and its effect on cortical rigidity.   Therefore, the experiments de-

TABLE II

Nuclear breakdown and cortical rigidity

% eggs with cortical granules moved at 2–3 minutes	% eggs showing nuclear breakdown at 8 minutes	Centrifugal force in gravities
28	25	4150
42	40	9340
10	9	2335
10	11	2335

scribed in Table II—in which all the eggs had intact nuclei at 2–4 minutes—were regarded as significant and indicated that those eggs in which the nucleus was destined to break down had a less rigid cortex.

A liquefaction of the protoplasmic cortex following the fertilization of Chaetopterus eggs has been reported by Wilson (1951).   Moser (1939) has also described cortical changes following stimulation of Arbacia eggs.   Wilson and Heilbrunn (1952) have interpreted these cortical changes to be a reflection of the release of calcium ions from binding to the cortical proteins and this, of course, would alter the consistency of the protoplasmic gel there.   In this study it appears that the liquefaction of the cortex occurs as a necessary prelude to nuclear breakdown and the next sections will show what role calcium plays in the process.

Role of calcium

That calcium plays a significant role in the activation of eggs has been shown by a considerable amount of work.   Perhaps the most important was the contribution

---

[4] This solution was made up as follows: 94 gms. NaCl, 20 gms. MgCl$_2$, 15.7 gms. Na$_2$SO$_4$, 2.7 gms. KCl, 0.77 gm. NaHCO$_3$, 0.1 gm. H$_3$BO$_4$, and 3983 gms. H$_2$O.   Eggs shed into this solution produced only about 10–40% nuclear breakdown.   These intermediate numbers (rather than 0% or 100%) permitted a basis for comparing the frequency of cortical change with the frequency of nuclear breakdown.   The inhibition was reversible, and so the solution proved to be a useful tool in this instance.

of Heilbrunn and Wilbur (1937). They demonstrated that the artificial induction of nuclear breakdown in Nereis eggs by ultraviolet light, NaCl or KCl was dependent on the presence of ionic calcium in the cell. If the eggs were placed in sodium citrate solutions, nuclear breakdown would not occur, citrate presumably making calcium unavailable for cellular activity. Repetition and extension of this work with Chaetopterus eggs was felt to be in order, since with this material no artificial measures are necessary for activation and, possibly, a different type of mechanism is at work.

Eggs were shed directly into a citrate solution (see Methods). This solution inhibited nuclear breakdown in ca. 90% of the eggs. If, 6 or 7 minutes after shedding into citrate, some of the eggs were placed in normal sea water and some were placed in Ca- and Mg-free sea water (see Table I), nuclear breakdown occurred in 100% of the eggs in normal sea water while a substantial percentage of those eggs in Ca- and Mg-free sea water was inhibited (see Table III).

These results suggested, of course, that divalent cations were necessary within the egg for activation to occur. When, however, the experiment was repeated, this

TABLE III

Experiments indicating a requirement for divalent cations (Ca and Mg)

Eggs shed directly into normal sea water	Eggs shed directly into Ca- and Mg-free sea water	Eggs shed directly into citrate and then, at 6–7 minutes, transferred to:	
		Normal s. w. or	Ca- and Mg-free s. w.
100	100	100	40
100	96	100	18
100	99	100	7

(Figures indicate % eggs showing nuclear breakdown.)

time placing the eggs (from citrate) either into normal sea water or Ca-free sea water (Mg present), it was observed that again all the eggs in normal sea water produced nuclear breakdown, while there was a high percentage of inhibition of those eggs in Ca-free sea water as seen in Table IV. Apparently Mg is not the necessary cation since it is supplied in the Ca-free sea water. Therefore, it would appear that Ca is the important factor.

A third series of experiments (Table V), in which Mg-free sea water (containing Ca) was used instead of Ca-free sea water, proved to be conclusive. The results of these experiments showed no difference in the percentage of nuclear breakdown of the eggs when they were removed from citrate to either normal sea water or Mg-free sea water. Indeed, nuclear breakdown occurred in 100% of the citrated eggs in both Mg-free sea water and normal sea water. This experiment confirms the view that Ca and not Mg is important for the mechanism responsible for the dissolution of the nucleus.

The results of these three series of experiments (as well as a considerable amount of unpublished work) amply demonstrate the need for Ca in the mechanism of nuclear breakdown in the cell, and are in agreement with the work of Heilbrunn and Wilbur (1937). (The role of Ca in the activation of cells is discussed by Heibrunn,

TABLE IV

Experiments indicating a requirement for calcium ions

Eggs shed directly into normal sea water	Eggs shed directly into Ca-free sea water	Eggs shed directly into citrate and then, at 6–7 minutes, transferred to:	
		Normal s. w. or	Ca-free s. w.
99	58*	100	6
100	52*	100	7
100	81*	100	24

(Figures indicate % eggs showing nuclear breakdown.)

* The controls in Ca-free sea water (without prior citrate treatment) were also inhibited (nuclear breakdown: 52–81%), but not to the extent that the eggs treated with citrate were (nuclear breakdown: 6–24%). It was shown (unpublished experiments done in another connection) by more conclusive means that, if 0.53 M NaCl were used instead of Ca-free sea water (right hand column of Table IV) calcium was again necessary for nuclear breakdown in eggs pre-treated with citrate—whereas the controls in NaCl (without prior citrate treatment) showed 100% activation. Apparently the inhibitory effect of Ca-free sea water (without prior citrate treatment) here was due to some type of ion antagonism.

and his book (1952) should be consulted for a review of his theory.) Lindahl (1937) has questioned the results of Heilbrunn and Wilbur, claiming that the inhibition by citrate could very well be due to acidity. However, the work reported here shows that, following citrate treatment, the eggs may be placed in almost any environment lacking Ca and still be inhibited. It is only when Ca is returned to the cell that activation occurs and, consequently, the inhibition can hardly be attributable to a pH effect.

Sequence of steps in the activation mechanism

In view of the fact that CO_2 (Goldstein, 1950) and sodium citrate both reversibly inhibit nuclear breakdown, it was decided to investigate the possibility of a stepwise mechanism in which the inhibitors might be acting on different stages in the response mechanism.

A series of experiments was carried out in which the eggs were first placed directly from the ovary either into normal sea water saturated with CO_2 or into a

TABLE V

Experiments indicating no requirement for magnesium ions

Eggs shed directly into normal sea water	Eggs shed directly into Mg-free sea water	Eggs shed directly into citrate and then, at 6–7 minutes, transferred to:	
		Normal s. w. or	Mg-free s. w.
100	91	100	100
100	98	100	100
100	100	100	97

(Figures indicate % eggs showing nuclear breakdown.)

citrate solution, and then, after 8–10 minutes in one of these solutions, were removed to the other inhibiting solution. Examination showed that nuclear breakdown would be prevented no matter in what sequence the inhibitors were applied. These facts in themselves give virtually no insight into the mechanism of activation, though the information obtained was useful for later interpretations.

A further series of seven experiments proved to be more revealing. If the eggs were shed initially either into 0.53 M NaCl (non-inhibitory) or into normal sea water, allowed to remain for three minutes (at 21° C.) and then removed either to normal sea water saturated with CO_2 or to a citrate solution, nuclear breakdown was inhibited only in those eggs in CO_2-saturated sea water.

The results of these experiments suggested a tentative hypothesis which was substantially confirmed and elaborated by later experiments. The data suggest that calcium must perform its (yet to be described) function during the first three minutes after the initial stimulus and that after this function is fulfilled, calcium is no longer needed—since citrate can no longer inhibit. Allen (personal communication) has also shown, in Spisula eggs, that the activation process is sensitive to the lack of calcium during the first four or five minutes after the stimulus is applied. The inhibition by CO_2, both by immediate immersion and immersion after three minutes, presented a more puzzling problem—though not an insoluble one. The immediate inhibition can be explained in at least two ways. CO_2 could either inhibit the release of calcium from binding (possibly in the cortex) or it might prevent calcium from performing any function once it had been released. (It is doubtful that CO_2 inhibits calcium release. In fact, later data indicate just the opposite—that CO_2 produces a release of calcium from binding with protein, for it is expected that in the presence of a sufficient concentration of CO_2 the protein involved is on the acid side of its isoelectric point and is probably no longer capable of binding cations.) Furthermore, CO_2 would appear to inhibit some step in the process that follows both calcium release and the immediate effect that such released calcium produces. This is evident from the fact that calcium apparently completes its function during the first three minutes following the initial stimulus and yet CO_2 is still capable of inhibiting after this time. (These relationships will be more clearly demonstrated by the evidence presented in the next section.) The release of calcium that has been mentioned has not been demonstrated directly for the Chaetopterus egg, though it has been shown for other forms (Mazia, 1937 and Örström and Örström, 1942). The evidence for the existence of this phenomenon in Chaetopterus eggs has been shown indirectly by Wilson and Heilbrunn (1952) and is suggested here by the time relationships of cortical liquefaction (at about two minutes after the eggs have been shed) and the functioning of calcium (at about three minutes after the eggs have been shed).

After the above experiments were performed, a serious criticism came to mind. If there were a significant difference in the rate of penetration of citrate and CO_2 into the cells, many of the conclusions based on the data (regarding time of action) from this entire section would not be valid. For example, the fact that CO_2 could inhibit nuclear breakdown following three minutes immersion in sea water, while citrate could not, might be a result of the ability of CO_2 to penetrate more rapidly and thus affect a reaction that was still sensitive; whereas if the citrate were slower in entering the cell, the reaction might be complete before citrate could be effective.

Fortunately, however, later experiments (see next section) indicate that the general conclusions are substantially correct.

Evidence for the existence of a calcium-activated component

In the previous section, the suggestion was made that after calcium is released from the cortex and during the first three minutes after the eggs leave the ovary, some factor or system—not well defined—was acted upon (or activated) by calcium. This view was based on some indirect evidence. However, as was intimated, some much more conclusive data are available to establish the fact that calcium activates another component.

The experiments to be described were made feasible by an earlier observation that if the eggs were shed into a solution of 0.53 M NaCl saturated with CO_2, not only would nuclear breakdown be inhibited but if the eggs were left in the solution long enough, the calcium of the eggs would leak out. Presumably CO_2 caused calcium to be released from binding with protein, and calcium ions were then able to diffuse out of the cell to a region of lower concentration, there being none in the external medium.

(Examination of Figure 1 will be helpful in understanding the description of the experiments to follow.) Two parallel series of experiments were run. In the first, eggs were shed directly into CO_2-saturated 0.53 M NaCl and allowed to remain in that solution for 12 minutes, during which time nuclear breakdown was inhibited and presumably the calcium of the eggs diffused out into the environment. After 12 minutes some of the eggs were transferred to 0.53 M NaCl (dish A in Fig. 1) and some other eggs were transferred to a solution made up of 7 parts of 0.53 M NaCl and 3 parts of 0.30 M CaCl$_2$ (dish B). If the idea presented above is correct, then nuclear breakdown could not occur in dish A—calcium having been lost before activation was effected. On the other hand, nuclear breakdown would be expected to occur in dish B since calcium was returned to the cell and again made available for activation. In the parallel experiment, eggs were shed directly into 0.53 M NaCl and allowed to remain in that solution for three minutes, during which time calcium would be released and functioning. After three minutes the eggs were placed in CO_2-saturated 0.53 M NaCl, as in the above experiment, and nuclear breakdown was inhibited during the time that the eggs remained in that solution. The eggs were left in this latter solution for 12 minutes, during which time, again, the calcium should have leaked out of the cells. At the end of the 12-minute period some of the eggs were placed in 0.53 M NaCl (dish C) and other eggs were placed in a solution composed of 7 parts of 0.53 M NaCl and 3 parts of 0.30 M CaCl$_2$ (dish D). According to the hypothesis, nuclear breakdown is expected to occur in both latter instances with calcium present or not, since calcium activation should have occurred during the first three minutes in 0.53 M NaCl. Therefore, it is expected that nuclear breakdown would result in both dishes C and D. The predictions for dishes A, B, C, and D were realized. In a typical experiment (in all, four experiments were performed) the following results were obtained:

Dish	% nuclear breakdown
A	22
B	98
C	93
D	88

The critical feature to note is the difference between A and C, though both were devoid of calcium. This evidence is considered as demonstrating that calcium participates in the activation of some component during the first three minutes following the release of the egg from the ovary, and that this calcium-activated factor no longer requires the presence of calcium as it acts more directly on the nucleus. (The process is in this way analogous to the activation of prothrombin to thrombin in blood clotting—a reaction which is also dependent on the presence of calcium. Also, as in the cellular process above, following the activation of thrombin, calcium
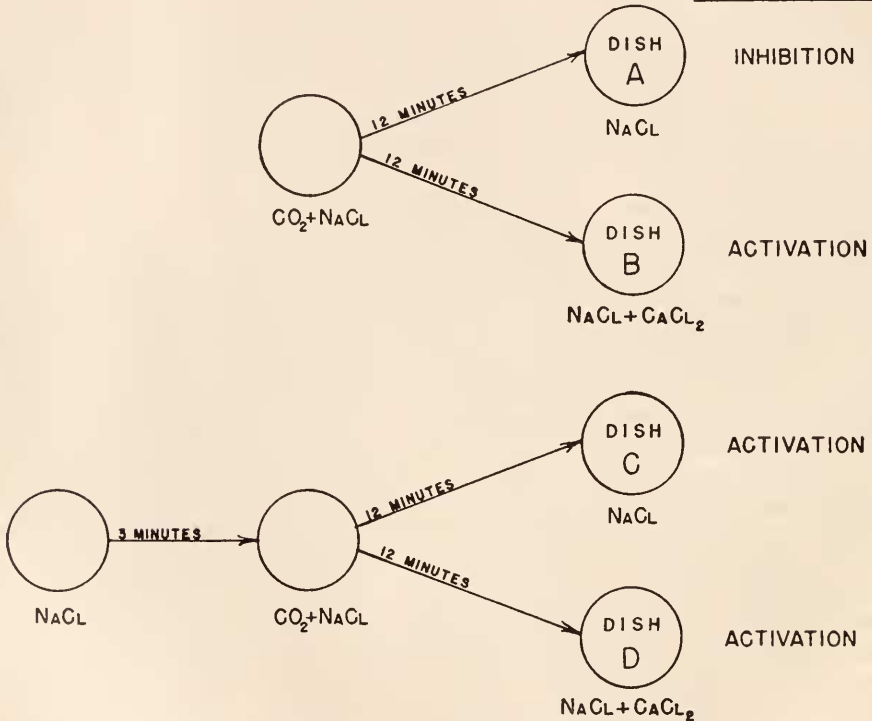

FIGURE 1. Experiment showing the existence of a calcium-activated component. See text for experimental details.

may be removed from the medium without inhibiting the subsequent reactions in the clotting process.)

It can be seen that calcium functions at approximately the same time, or shortly after, the cortex loses its rigidity. This evidence suggests that the two phenomena are closely associated and that, presumably, calcium is released from binding in the protoplasmic cortex—resulting in the loss of rigidity. Admittedly, it is impossible to state unequivocally that the *released* calcium is responsible for the activation resulting in the breakdown of the nucleus, since it is possible that the amount of free calcium ions present before stimulation was adequate for nuclear breakdown to have

occurred.   However, there is probably very little free calcium present in the unactivated egg protoplasm.   This is certainly true for the sea urchin egg (Mazia, 1937).

Nature of the calcium-activated component

Since the calcium-activated factor may be acting directly upon the nuclear membrane, which is presumably protein or lipoprotein in character, at least three possibilities as to the nature of the factor were envisioned. (1) Conceivably the activated factor could be acting as a reducing agent to convert S-S linkages to -SH radicals in the protein, thereby leading to a dissolution of the architecture of the membrane.   Experiments utilizing -SH and S-S compounds, as well as reagents affecting these groups, established that this probably could not be the mechanism operating.   Several reagents were used but most of the experiments were performed with glutathione, cystine and p-chloromercuribenzoic acid, none of which behaved as would be expected from the above hypothesis.   (2) It is possible, assuming the presence of lipid in the membrane, that a lipase digests part of the structure and thus breakdown of the nucleus results.   This possibility has not been tested.   (3) The most appealing hypothesis appears to be that the calcium-activated factor is a proteolytic enzyme.

The foundations for the third view are as follows:

(a) Calcium is known to activate, or participate in the activation of, some proteases.   (However, this also applies to some other enzymes, including lipases.)

(b) The nuclear membrane is probably mostly protein and appears to be dissolved during maturation.

(c) Yamane (1930) has reported some experiments which suggest a protease participation in some phase of the maturation mitosis of mammalian ova.   Bohus Jensen (1948) and others have also described a certain degree of "nuclear activation" of sea urchin eggs by commercial trypsin.

a. Behavior of trypsin

In order to ascertain if a proteolytic enzyme is capable of producing nuclear breakdown, experiments were carried out utilizing crystalline trypsin (obtained from Worthington Biochemical Laboratory).

(1) Eggs were shed directly into a citrate solution in order to remove the calcium from the cells.   After 8 minutes in that solution, some of the eggs were placed in a 0.02% trypsin solution made up in Ca-free sea water, and some eggs were placed in Ca-free sea water without trypsin.   The first four items in Table VI show the results of this type of experiment.   In every case, though nuclear breakdown took as long as 20–25 minutes for some eggs as compared to the normal time of 7–8 minutes, the eggs which were placed in the trypsin solution gave a substantially higher percentage of nuclear breakdown—though no significant amount of calcium was present in the eggs.

(2) As a check against any possible contamination of the trypsin preparation, another set of experiments was performed in which a trypsin inhibitor was used. In these experiments, following the immersion in citrate for 8 minutes, some of the eggs were transferred to Ca-free sea water, some of the eggs were transferred to 0.03% trypsin in Ca-free sea water, and some were placed in 0.03% trypsin in Ca-free sea water to which was added crystalline soybean trypsin inhibitor (obtained

from Worthington Biochemical Laboratory) to make a 0.03% concentration of that substance. The results of this experiment are shown in the last four items in Table VI. It can be seen, from the figures in the right hand column, that the inhibitor completely blocked the action of trypsin in producing nuclear breakdown. Apparently, then, it was the action of the enzyme itself which was responsible for the breakdown of the nucleus.

(3) Since trypsin is a fairly large molecule, there was some doubt as to whether the enzyme was actually entering the cell. Possibly trypsin was simply acting on the surface to activate, or release, some factor which would act more directly on the nuclear membrane. To determine if trypsin actually penetrates into the cell, trypsin solutions were made up in acid Ca-free sea water (pH 3.5–4.0) and then tested. Following the same procedures as in (1) above, three experiments showed that eggs placed in trypsin in acid media produced substantially as high a percentage

TABLE VI

Effect of trypsin on nuclear breakdown

Exper. No.	Concentration of trypsin	% Eggs showing nuclear breakdown in:		
		Ca-free sea water	Trypsin	Trypsin and inhibitor
1	0.02%	31	49	
2	0.02%	5	34	
3	0.02%	5	57	
4	0.02%	4	37	
5	0.03%	10	65	8
6	0.03%	8	81	10
7	0.03%	2	26	3
8	0.03%	9	34	10

of nuclear breakdown as those eggs placed in trypsin in ordinary Ca-free sea water (pH 8.0–8.2). Since, under these circumstances, trypsin is effective at an external pH at which it can not perform proteolysis, the data suggest that the enzyme is entering the cells where the cytoplasmic pH is relatively unaffected by the external hydrogen ion concentration.

Thus we see that trypsin can act within the cell to produce nuclear breakdown in the *absence* of calcium. Consequently, the enzyme appears to be performing the same function as the aforementioned calcium-activated component. Encouragement for these views is offered by the recent observation of Callan (1952) that trypsin is capable of dissolving the membrane of isolated nuclei of Triturus oöcytes.

b. Protease of the eggs

Encouraged by the results of the trypsin experiments, attempts were made to isolate and characterize the intracellular protease—if present. The results to late are preliminary and should only be considered as suggestive

All the eggs from approximately 5 worms were shed into about 35 ml. of Ca free sea water—the process required about four minutes. After mixing to insure equal distribution, the suspension was divided into two parts of 15 ml. each. One

portion was frozen immediately in a bath of solid CO_2 and methyl Cellosolve (at ca. $-75°$ C.). The eggs in the other fraction were centrifuged and re-suspended in 15 ml. of normal sea water and allowed to stand for another 8 minutes, after which time (about 14 minutes after the initial shedding) that suspension was also frozen. The two preparations were then dehydrated by freeze-drying. The dried preparations were re-suspended in 5 ml. of distilled water each and allowed to stand for two hours in a refrigerator at $6°$ C. After two hours in the refrigerator, the suspensions were centrifuged and the supernatants saved.

The two supernatants were then tested for proteolytic activity by the method of Anson (1938). Four minutes after the Folin-Ciocalteau color reagent was added. the optical density, at 700 mμ, was determined with a Coleman spectrophotometer. The results of two experiments are given below; data are expressed in arbitrary units:

	Preparation	Proteolytic activity
	Frozen early	11
Experiment I		
	Frozen late	$- 2$
	Frozen early	19
Experiment II		
	Frozen late	$- 2$

While the experiments are only preliminary, some interesting possibilities are suggested. It appears that the extracts of the eggs that were frozen about 5 minutes after shedding have a substantial proteolytic activity. This activity would result, presumably, in part from activation before freezing and in part from activation of the dried preparation upon re-suspension in water—the calcium content of the egg material probably being sufficient to produce some protease activation. On the other hand those eggs which had been frozen about 14 minutes after shedding had virtually no activity—the negative figures may be insignificant. The lack of activity in this case can be regarded as the result of a natural protective mechanism of the cell, which would come into play to block further proteolytic digestion before it could act on components of the cell other than the nuclear membrane. This could prevent a possible autolysis. Heilbrunn (1952, p. 654) has considered this type of eventuality and suggests that the phenomenon may be the result of some sort of cellular homeostasis. Thus, in this particular instance it is possible that, following nuclear breakdown, the activated protease could liberate heparin, or a like substance, from binding to protein. (The feasibility of this mechanism has been demonstrated by *in vitro* experiments utilizing trypsin to free heparin from a complex with protamine (Kelly, 1951).) This heparin-like compound would then, presumably, be capable of blocking further proteolysis, and this idea finds support from the evidence showing that heparin is capable of preventing digestion of proteins by trypsin (Glazko and Ferguson, 1940). Further support of this hypothesis is offered by some *in vivo* observations of Kelly (1950). Kelly, by means of metachromatic staining with toluidine blue, has found that there are "granules" of heparin-like material present in Chaetopterus eggs following nuclear breakdown but he was unable to detect their presence before the dissolution of the nucleus (personal communication).

It has been difficult, thus far, to obtain a suitable extract of the eggs within the first minute after shedding. This is regrettable, since the theory holds that the eggs should have little or no proteolytic activity before calcium activation has occurred and it would be desirable to test this. Nevertheless, if we assume that the calcium-activated factor behaves as postulated here, it would not be conclusive proof that this was the system leading to nuclear breakdown in the cell. Direct proof would come from a demonstration that the isolated factor could act on its "natural" substrate—namely, the isolated nuclei. The few experiments that have been performed with this in mind have yielded no information since it has been impossible, as yet, to isolate the nuclei in any condition approaching a reasonably normal physiological state.

Conclusions

From the evidence presented here, the following conclusions have been provisionally drawn regarding the process of activation and nuclear breakdown in the Chaetopterus egg:

1. Within the animal the eggs are "primed" for activation, but some inhibitor is present in the ovarian environment which presumably blocks the release of calcium from the protoplasmic cortex.

2. Following the liberation of the eggs from the animal, calcium ions are released —probably from binding with protein or lipoprotein in the cortex of the cell.

3. Within three minutes after the initial stimulus the free calcium ions activate a system, which is thought to involve a proteolytic enzyme.

4. This activated system then acts directly on the nucleus and produces a dissolution of the nuclear membrane.

5. Immediately following nuclear breakdown some protective mechanism comes into play to prevent excess autolysis of the cell, and probably to allow the cyclic events of mitosis to continue.

The above ideas probably represent the simplest possible mechanism that could be operating.

Two critical problems deserve the earliest consideration. First, it is desirable to achieve a better characterization of the calcium-activated system. Second, a verification of the hypothesis requires that we determine whether or not the isolated system will act on isolated nuclei.

Summary

1. A study has been made of activation and nuclear breakdown in Chaetopterus eggs in an effort to elucidate the various steps in the response mechanism.

2. Within two minutes after the immature egg is stimulated there is a liquefaction of the protoplasmic cortex, which occurs as a prelude to the breakdown of the nucleus. The cortical change appears to be associated with a release of calcium ions from that region.

3. Within three minutes after the initial stimulus calcium ions activate a system which acts more directly on the nucleus and which, having once been activated, no longer requires the presence of calcium ions.

4. It was postulated that the system activated by calcium involved a proteolytic enzyme. To test this possibility experiments were run utilizing crystalline trypsin. It was found that trypsin could produce nuclear breakdown in the absence of calcium and that this activity was completely blocked by soybean trypsin inhibitor. Furthermore, when eggs were placed in trypsin solutions made up in acid media (pH 3.5–4.0), nuclear breakdown still occurred, indicating that the enzyme was penetrating into the interior of the cell.

5. Preliminary experiments suggest that there is a protease in the eggs which behaves as predicted. In addition, it appears that once nuclear breakdown is effected, some mechanism comes into play to block further action of the enzyme.

LITERATURE CITED

ALLYN, H. M., 1912. The initiation of development in Chaetopterus. *Biol. Bull.*, **24** : 21–72.

ANSON, M. L., 1938. The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.*, **22** : 79–91.

BOHUS JENSEN, A., 1948. Activation of unfertilized sea urchin eggs by trypsin. *Nature,* **162** : 931–932.

CALLAN, H. G., 1952. A general account of experimental work on Amphibian oocyte nuclei. *Symposia Soc. Exp. Biol.*, **6** : 243–255.

DALCQ, A., 1928. Le rôle du calcium et du potassium dans l'entreé en maturation de l'oeuf de Pholade (*Barnea candida*). *Protoplasma*, **4** : 18–44.

DANIELLI, J. F., 1951. Chapter IV in: Cytology and Cell Physiology, G. H. Bourne, ed. Oxford University Press, Oxford.

GLAZKO, A. J., AND J. H. FERGUSON, 1940. Inhibition of tryptases by heparin. *Proc. Soc. Exp. Biol. Med.*, **45** : 43–46.

GOLDSTEIN, L., 1950. An experimental study of maturation in Chaetopterus eggs. *Biol. Bull.*, **99** : 341.

HEILBRUNN, L. V., 1952. An outline of general physiology. Third edition. W. B. Saunders Co., Philadelphia.

HEILBRUNN, L. V., AND K. M. WILBUR, 1937. Stimulation and nuclear breakdown in the Nereis egg. *Biol. Bull.*, **73** : 557–564.

HÖRSTADIUS, S., 1923. Physiologische Untersuchungen über die Eireifung bei *Pomatocerus triqueter L. Arch. mikroskop. Anat. Entw.*, **98** : 1–9.

KELLY, J. W., 1950. The localization of a metachromatic substance in the Chaetopterus egg. *Protoplasma*, **39** : 386–388.

KELLY, J. W., 1951. Effects of x-ray and trypsin on the metachromasy of heparin-basic protein combinations. *Biol. Bull.*, **101** : 223.

LEFEVRE, P. G., 1945. Certain chemical factors influencing artificial activation of Nereis eggs. *Biol. Bull.*, **89** : 144–156.

LILLIE, R. S., 1941. Further experiments on artificial parthenogenesis in starfish eggs, with a review. *Physiol. Zool.*, **14** : 239–267.

LINDAHL, P. E., 1937. Physiologisch-chemische Probleme der Embryonalentwicklung. *Fortschritte der Zoologie*, **3** : 271–305.

MAZIA, D., 1937. The release of calcium in Arbacia eggs on fertilization. *J. Cell. Comp. Physiol.*, **10** : 291–304.

MOSER, F., 1939. Studies on a cortical layer response to stimulating agents in the Arbacia egg. I. Response to insemination. *J. Exp. Zool.*, **80** : 423–445.

ÖRSTRÖM, A., AND M. ÖRSTRÖM, 1942. Über die Bindung von Kalzium in Ei und Larve von *Paracentrotus lividus. Protoplasma*, **36** : 475–490.

PASTEELS, J. J., 1935. Recherches sur le déterminisme de l'entrée en maturation de l'oeuf chez divers Invertébrés marins. *Arch. de Biol.*, **46** : 229–262.

RUNNSTRÖM, J., 1949. The mechanism of fertilization in metazoa. *Advances in Enzymology,* **9** : 241–327.

SCHEER, B. T., AND M. A. R. SCHEER, 1947. Some interrelationships of drug and ion actions in the artificial activation of marine eggs. *Physiol. Zool.,* **20**: 15–32.

SCOTT, A. C., AND G. LeBARON, JR., 1950. Factors involved in the breakdown of the germinal vesicle in the egg of *Chaetopterus pergamentaceus.* *Biol. Bull.,* **99**: 363.

TYLER, A., 1941. Artificial parthenogenesis. *Biol. Rev.,* **16**: 291–336.

WATTENBERG, H., 1938. Zur Chemie de Meerwassers. Über die in Spuren vorkommenden Elemente. *Zeitschr. f. anorg. u. allgem. Chem.,* **236**: 339–360.

WILSON, W. L., 1951. The rigidity of the cell cortex during cell division. *J. Cell. Comp. Physiol.,* **38**: 409–415.

WILSON, W. L., AND L. V. HEILBRUNN, 1952. The protoplasmic cortex in relation to stimulation. *Biol. Bull.,* **103**: 139–144.

YAMANE, J., 1930. The proteolytic action of mammalian spermatozoa and its bearing upon the second maturation division of ova. *Cytologia,* **1**: 394–403.